

The GGAs Promote ARF-Dependent Recruitment of Clathrin to the TGN

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Summary

The GGAs constitute a family of modular adaptor-related proteins that bind ADP-ribosylation factors (ARFs) and localize to the trans-Golgi network (TGN) via their GAT domains. Here, we show that binding of the GAT domain stabilizes membrane-bound ARF1-GTP due to interference with the action of GTPase-activating proteins. We also show that the hinge and ear domains of the GGAs interact with clathrin *in vitro*, and that the GGAs promote recruitment of clathrin to liposomes *in vitro* and to TGN membranes *in vivo*. These observations suggest that the GGAs could function to link clathrin to membrane-bound ARF-GTP.

Introduction

The GGAs (Golgi-associated, γ -adaptin homologous, ARF-interacting proteins) constitute a family of proteins that associate with the cytoplasmic face of the trans-Golgi network (TGN) and interact with ADP-ribosylation factors (ARFs) (Black and Pelham, 2000; Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000; Poussu et al., 2000; Takatsu et al., 2000). Three GGAs have been identified in humans (GGA1, GGA2, and GGA3), two in *S. cerevisiae* (Gga1p and Gga2p), and one each in *C. elegans* and *D. melanogaster*. Immunoelectron microscopy analyses have shown that the human GGAs localize to electron-dense coats associated with TGN membranes (Dell'Angelica et al., 2000; Hirst et al., 2000). Association of the GGAs with the TGN is regulated by ARFs (Boman et al., 2000; Dell'Angelica et al., 2000; Hirst et al., 2000; Zhdankina et al., 2001), which also control membrane recruitment of coat proteins such as COPI, AP-1 (adaptor protein 1), AP-3, and AP-4. Disruption of both GGA genes in *S. cerevisiae* results in impaired sorting of carboxypeptidase Y (Dell'Angelica et al., 2000; Hirst et al., 2000; Zhdankina et al., 2001) and Pep12p (Black and Pelham, 2000) from the late Golgi complex to the vacuole, the yeast counterpart of the mammalian lysosome. Taken together, these observations suggest that the GGAs are components of protein coats involved in protein transport from the TGN to the endosomal-lysosomal system. The exact nature of the

coats containing GGAs, however, remains to be elucidated.

The domain organization of the GGAs may hold clues to the mechanisms by which GGA-containing coats are assembled onto membranes, as well as to the identity of other components of such coats. The GGAs are organized into four domains herein referred to as VHS, GAT, hinge, and γ -adaptin ear (GAE) (Figure 1, scheme). The GAT domain is directly responsible for binding of the GGAs to several ARF family members including ARF1 (Dell'Angelica et al., 2000) and ARF3 (Boman et al., 2000). These ARFs cycle between a GTP-bound form that is associated with Golgi membranes and a GDP form that is cytosolic (reviewed by Donaldson and Jackson, 2000). The ARF cycle is regulated by guanine nucleotide exchange factors (GEFs) that promote the exchange of GTP for GDP, and GTPase-activating proteins (GAPs) that induce hydrolysis of GTP bound to ARFs (reviewed by Donaldson and Jackson, 2000). The GAT domain specifically interacts with the GTP-bound form of ARF1 (Dell'Angelica et al., 2000) and ARF3 (Boman et al., 2000), suggesting that the GGAs may function as ARF effectors. Overexpression of the GAT domain displaces other ARF1-regulated coats from membranes, probably due to sequestration of ARF1-GTP (Dell'Angelica et al., 2000). In addition, the GAT domain of GGA3 is able to target a reporter protein to the TGN (Dell'Angelica et al., 2000). It is currently unknown whether the ARF1 binding and TGN-targeting activities are manifestations of a single underlying property of the GAT domain. Even less is known about the function of the other domains of the GGAs, although the hinge domains have been noted to contain sequences resembling clathrin binding motifs (Dell'Angelica et al., 2000).

In the present study, we have conducted a structure-function analysis of the GAT and hinge domains aimed at elucidating the nature of GGA-containing coats. We find that binding of the GAT domain interferes with the action of GAPs on ARF1 *in vitro*, causing stabilization of ARF1-GTP. In line with this observation, overexpression of the GAT domain stabilizes the association of ARF1-GTP with TGN membranes *in vivo*. These observations imply that GGAs and GAPs compete for interaction with ARF1-GTP and suggest a novel model for ARF1 regulation in which effectors delay dissociation of ARF1 from Golgi membranes. We also show that the hinge domains of the three human GGAs bind clathrin and that GGA1 mediates GTP-dependent recruitment of clathrin to liposomes *in vitro* and to TGN membranes *in vivo*. Expression of a truncated GGA1 unable to bind clathrin, on the other hand, causes dissociation of clathrin from the TGN. These findings suggest that the GGAs could function to link clathrin to membrane-bound ARF-GTP.

Results

Delineation of Functional Determinants within the GAT Domain of the GGAs

To investigate whether the various activities ascribed to the GAT domain of GGA3 (i.e., Golgi targeting, dis-

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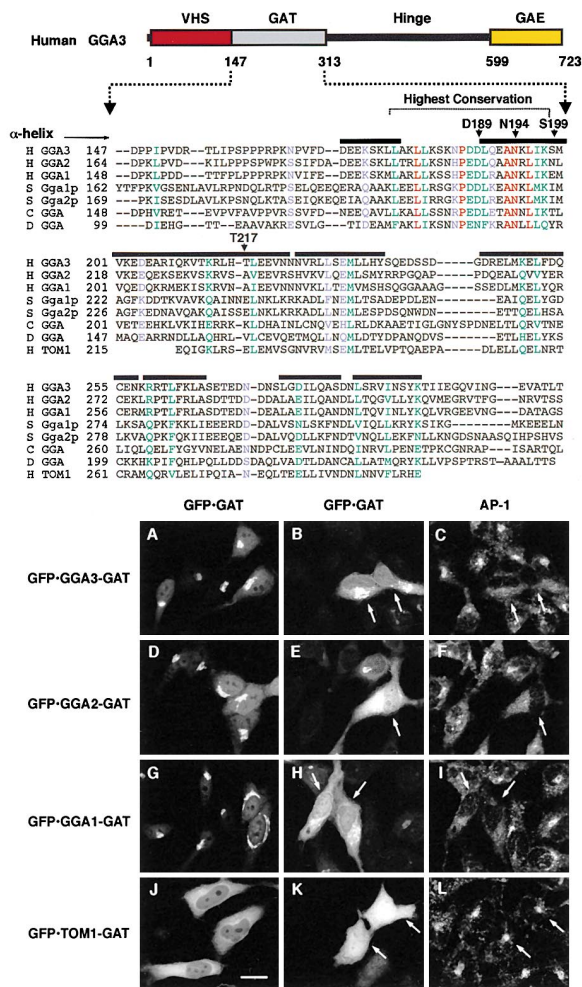


Figure 1. Conservation of GAT Domain Structure and Function

(Top) Schematic representation of the domain organization of human GGA3 and multiple sequence alignment of the GAT domains of human (H) GGA3 (AF219138), human GGA2 (A-735G6.4), human GGA1 (AF218584), *S. cerevisiae* (S) Gga1p (YDR358W), *S. cerevisiae* Gga2p (YHR108W), *C. elegans* (C) GGA (Z68014), *D. melanogaster* (D) GGA (A1061795), and human TOM1 (AJ006973). Red, identical residues; green, strongly conserved residues; and blue, weakly conserved residues. The region of highest conservation and the location of residues targeted for mutagenesis are indicated. Black lines denote regions of predicted α helices.

(Bottom) Intracellular localization of GFP fusion proteins containing the GAT domain of the three human GGAs and the TOM1 protein. HeLa cells were transfected with plasmids encoding GFP fused to GGA3-GAT (A–C), GGA2-GAT (D–F), GGA1-GAT (G–I), or TOM1-GAT (J–L). Cells were fixed, permeabilized, and examined by confocal fluorescence microscopy (A, D, G, and J) or immunostained with the 100/3 antibody to the γ 1-adaptin subunit of AP-1 followed by Cy3-conjugated rabbit anti-mouse IgG and analyzed for GFP fluorescence (B, E, H, and K) and γ 1-adaptin staining (C, F, I, and L). (A), (D), (G), and (J) show cells expressing low to moderate levels of the GFP fusion proteins. Arrows in (B), (C), (E), (F), (H), (I), (K), and (L) point to cells expressing high levels of the GFP fusion proteins. Immunoblot analysis of the transfected cells showed similar levels of expression for all the GFP fusion proteins (data not shown). Bar, 20 μ m.

placement of ARF-regulated coats, and ARF binding) are all functionally linked, we sought to delineate the regions and specific amino acid residues responsible

for each of those activities. The GAT domain spans \sim 150 amino acid residues (e.g., residues 147–310 of GGA3) and is predicted to comprise several α helices (indicated by solid lines in Figure 1 alignments). The highest degree of conservation among the GAT domains of GGAs from humans, *S. cerevisiae*, *C. elegans*, and *D. melanogaster* occurs within a 20 amino acid stretch spanning residues 179–199 of GGA3 (Figure 1 alignments). The GAT domain of the GGAs also exhibits a low degree of homology to a segment of the human proteins TOM1 (target of myb 1) and TOM1L1 (target of myb 1-like 1), but this homology starts downstream of the segment of highest conservation. The similarities and differences revealed by these sequence comparisons prompted us to analyze the Golgi targeting and AP-1-displacement activities of all the human GGAs and human TOM1.

As previously shown (Dell'Angelica et al., 2000), the GAT domain of human GGA3 was sufficient to target the reporter green fluorescent protein (GFP) to the Golgi complex at low to moderate expression levels (Figure 1A), and to cause dissociation of AP-1 from the Golgi complex at high expression levels (Figures 1B and 1C) (AP-3 behaved like AP-1 in these and all subsequent experiments; data not shown). The GAT domains of human GGA2 and GGA1 were found to possess the same activities (Figures 1D–1I). In contrast, the GAT domain of human TOM1 did not target GFP to the Golgi complex at any expression level (Figure 1J), nor displace AP-1 upon overexpression (Figures 1K and 1L). These observations pointed to the region of highest conservation, present in the three human GGAs but absent from TOM1, as a critical determinant of Golgi targeting and AP-1 displacement.

To investigate further the functional importance of different parts of the GAT domain, we examined the behavior of truncated GGA3-GAT constructs fused to GFP. The results of these experiments are summarized in the scheme in Figure 2. A construct spanning amino acids 170–233 of GGA3-GAT and encompassing the region of highest conservation was still targeted to the Golgi complex and displaced AP-1, albeit with reduced efficiency (indicated as \pm in Figure 2, top panel). Further trimming from either end of this construct resulted in loss of both activities. We then introduced point mutations within the 170–233 segment in the context of the GFP-GGA3-GAT construct and examined the activities of the resulting proteins. Mutation of the conserved D189 or N194 residues to alanine was predicted not to alter the local conformation of the protein, according to the NPS@ program (<http://www.ibcp.fr/predict.html>). Interestingly, mutation of D189 had no effect on either the Golgi targeting or AP-1 displacement activities of the GFP-GGA3-GAT construct (Figures 2A–2C), whereas mutation of N194 completely abolished both activities (Figures 2D–2F). Moreover, mutation of N194 in the context of full-length GGA3 precluded localization of this protein to the Golgi complex (Figures 2J–2K). These observations suggest that N194 is directly involved in the Golgi localization of both the GAT domain and full-length GGA3. To investigate whether the predominant α -helical character of the GAT domain was also important for function, we mutated two nonconserved amino acids, S199 and T217, to proline residues. These mutations were predicted by the NPS@ program to disrupt

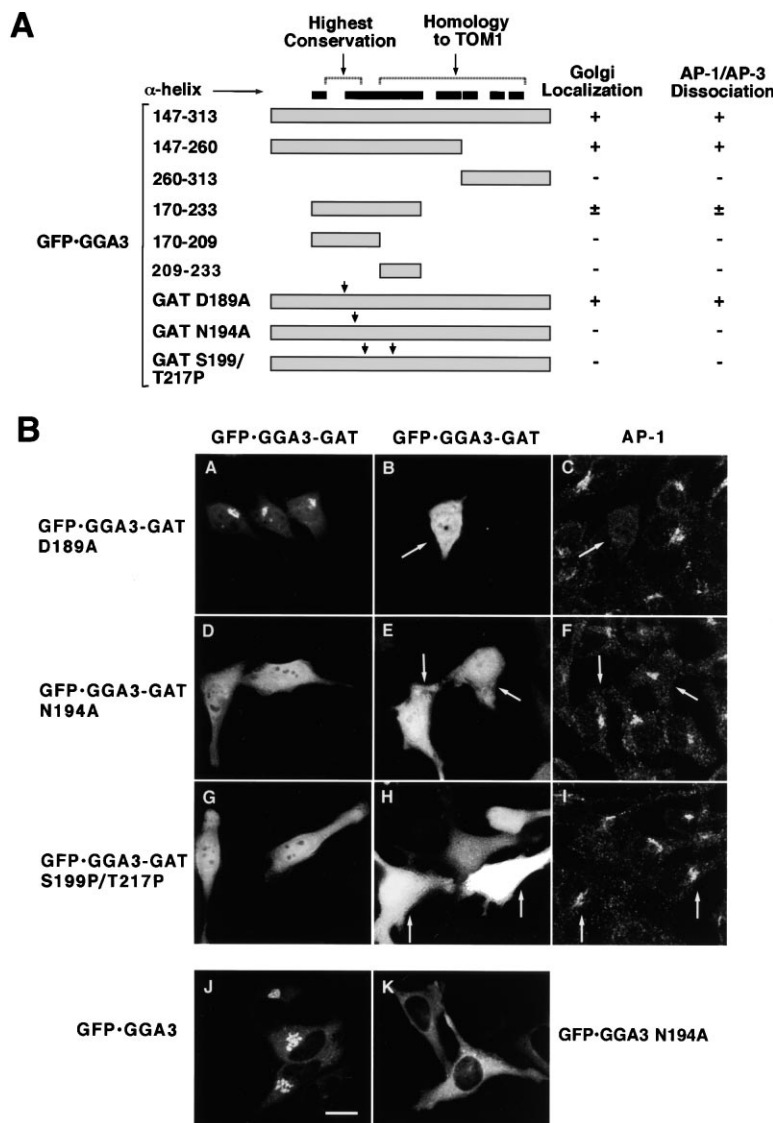


Figure 2. Mutational Analysis of the GAT Domain of Human GGA3

The mutants represented in (A) were transfected into HeLa cells and analyzed for Golgi localization at low to moderate expression levels (+, Golgi; -, cytosol) and effect on AP-1 and AP-3 distribution at high expression levels (+, Golgi/endosomes; -, cytosol). (B) Examples of some of the results summarized in (A). Effects of overexpression of several GAT domain mutants on the distribution of AP-1. HeLa cells were transiently transfected with plasmids encoding GFP-GGA3-GAT D189A (A-C), GFP-GGA3-GAT N194A (D-F), GFP-GGA3-GAT S199P/T217P (G-I), and analyzed at low (A, D, and G) and high (B, E, and H) levels of expression for GFP fluorescence and staining with the 100/3 antibody to γ 1-adaptin (C, F, and I). Arrows point to overexpressing cells. Plasmids encoding GFP-GGA3 (J) and GFP-GGA3 N194A (K) were also transfected into HeLa cells and analyzed for GFP fluorescence. Bar, 20 μ m.

the conformation of the longest α -helical stretch within the GAT domain (see schemes in Figures 1 and 2). We observed that these mutations also resulted in loss of Golgi localization and an inability to displace AP-1 (Figures 2G-2I). Thus, specific residues such as N194, as well as the overall conformation of the GAT domain, are critical for these two activities.

Structural Requirements for the Interaction of the GAT Domain with ARF1

In all of the experiments described above, we noticed a perfect correlation between the Golgi localization and AP-1 displacement activities of the different constructs, suggesting that both activities could arise from the ability of the GAT domain to bind ARF1. To investigate this possibility, we used the yeast two-hybrid system to assess interactions between various GGA3-GAT and ARF1 constructs. In these assays, we used the Q71L mutant of ARF1, which is locked in the GTP-bound state. In accord with the functional analyses described above, mutation of D189 in GGA3-GAT to alanine did not affect

the interaction with ARF1-Q71L, whereas mutation of N194 to alanine, or S199 and T217 to prolines, abolished the interaction (Figure 3). Mutation of N194 to alanine in the context of full-length GGA3 also abrogated interaction with ARF1 Q71L (Figure 3). Thus, the same structural features of GGA3-GAT that enable Golgi localization and AP-1 displacement are required for interactions with ARF1. We also mutated residues located in the switch 1 and switch 2 regions of ARF1, which change their conformation upon binding of GTP or GDP and thus serve as conditional binding sites for ARF effectors (reviewed in Roth, 1999). Mutations of either of two switch 1 residues (F51 to tyrosine and I49 to threonine, Kuai et al., 2000) in the context of ARF1-Q71L prevented interactions with the GAT domain of GGA3 (Figure 3). Mutation of a switch 2 residue (Y81 to histidine) had a partial effect (Figure 3). Taken together, these observations suggest that the GGAs are recruited to the Golgi complex by direct interaction with ARF-GTP. This interaction likely involves contacts between the highly conserved region of the GGA-GAT domains (including N194)

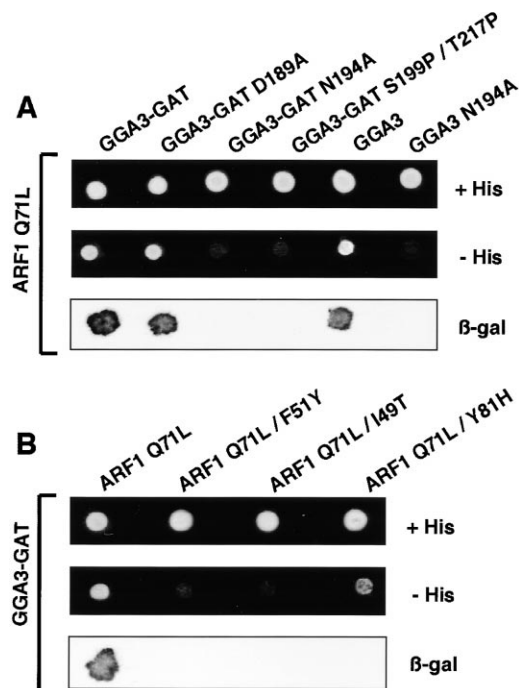


Figure 3. Two-Hybrid Analysis of the Interactions of Different ARF1 and GGA3-GAT Constructs

(A) Plate growth and β -galactosidase assays for interactions between constitutively activated ARF1 Q71L and several mutants of the GGA3-GAT domain (GGA3-GAT, GGA3-GAT D189A, GGA3-GAT N194A, GGA3-GAT S199P/T217P) or the full-length GGA3 protein (GGA3, GGA3 N194A).

(B) Interaction of GGA3-GAT with different ARF1 mutants (ARF1 Q71L, ARF1 Q71L/F51Y, ARF1 Q71L/I49T, and ARF1 Q71L/Y81H). Yeast transformants expressing the combination of constructs indicated in the figure were spotted onto plates lacking leucine and tryptophan, with or without histidine (+His and -His, respectively) in the presence of 5 mM 3AT. Filter β -galactosidase (β -gal) assays were performed on cells grown in the presence of histidine.

and the switch 1 and switch 2 regions of ARF1. The observed displacement of AP-1 from the Golgi complex by GAT must thus be due to competition for the same effector binding site on ARF1 or ARF3.

The GAT Domain Inhibits GAP-Induced GTPase Activity of ARF1 In Vitro

The detailed characterization of ARF-GGA interactions enabled us to analyze whether the GGAs modulate the ARF cycle. Since both GGAs and GAPs interact with the GTP-bound form of ARFs, we could readily assess whether their interactions are neutral, synergistic, or inhibitory. To this end, we examined the effect of different recombinant GGA constructs on the GTPase activity of ARF1 in the presence of ARF GAPs and phospholipids in vitro (Figure 4). Strikingly, addition of a VHS+GAT construct to the assay mixture inhibited GTP hydrolysis induced by two different ARF GAPs, ASAP1 (Brown et al., 1998) and ARF GAP1 (Cukierman et al., 1995) (Figure 4A). Unlike VHS+GAT, VHS alone had no effect on GTP hydrolysis (Figure 4B), pointing to the GAT domain as the source of the inhibitory activity. This was directly

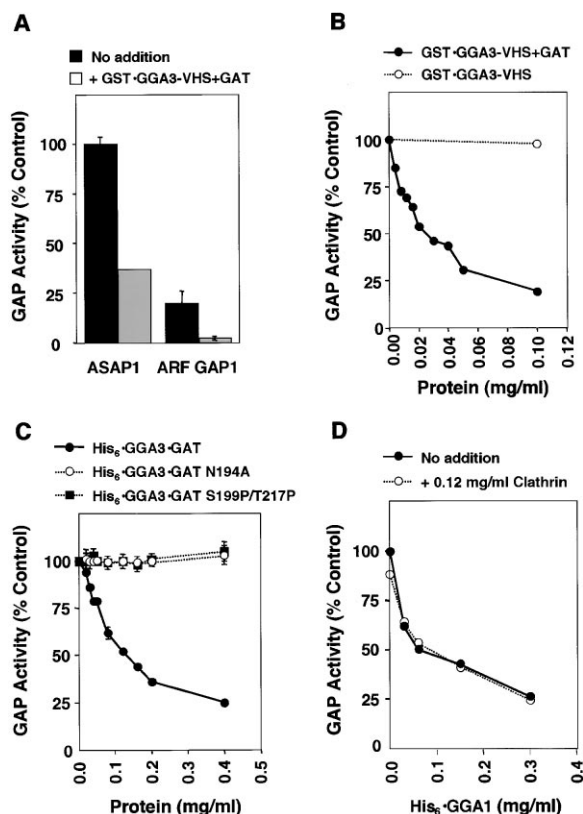


Figure 4. The GAT Domain Inhibits GAP-Induced GTPase Activity of ARF1 In Vitro

(A) GTP-hydrolysis of ARF1 in the presence of ASAP1 or ARF GAP1 was measured as described in Experimental Procedures in the absence (-) or presence (+) of GST-GGA3-VHS+GAT.

(B) Myristoylated ARF1 loaded with [α -³²P]GTP was assayed for GTP hydrolysis in the presence of ASAP1 and the indicated concentrations of GST-GGA3-VHS+GAT and GST-GGA3-VHS.

(C) GTP hydrolysis was measured as in (B) in the presence of His₆-GGA3-GAT, His₆-GGA3-GAT N194A, or His₆-GGA3-GAT S199P/T217P.

(D) GTP hydrolysis was measured as in (B) in the presence of varying concentrations of His₆-GGA1 (full length) and in the absence or presence of 0.12 mg/ml clathrin purified from bovine brain.

assessed by producing a recombinant GAT domain of GGA3, and this domain bearing the N194A or S199P/T217P mutations described above. Analysis of these recombinant proteins by circular dichroism spectroscopy confirmed the predictions that GAT has a high α -helical content, that the N194A mutation has no effect on the conformation of this domain, and that the S199P/T217P mutations alter its conformation (data not shown). The GAT construct alone was found to inhibit the GTPase activity of ARF1 in the presence of ASAP1, while the N194A and S199P/T217P mutant construct were completely inactive in this assay (Figure 4C). Finally, full-length His₆-tagged GGA1 was also found to inhibit GAP activity with a half-maximal value of 0.06 mg/ml (0.7 μ M), irrespective of the presence or absence of clathrin (see below) in the incubation mixture (Figure 4D). These observations suggest that binding of the GGAs via their GAT domains hinders the action of the GAPs on ARF1 and, as a consequence, stabilizes ARF1-GTP.

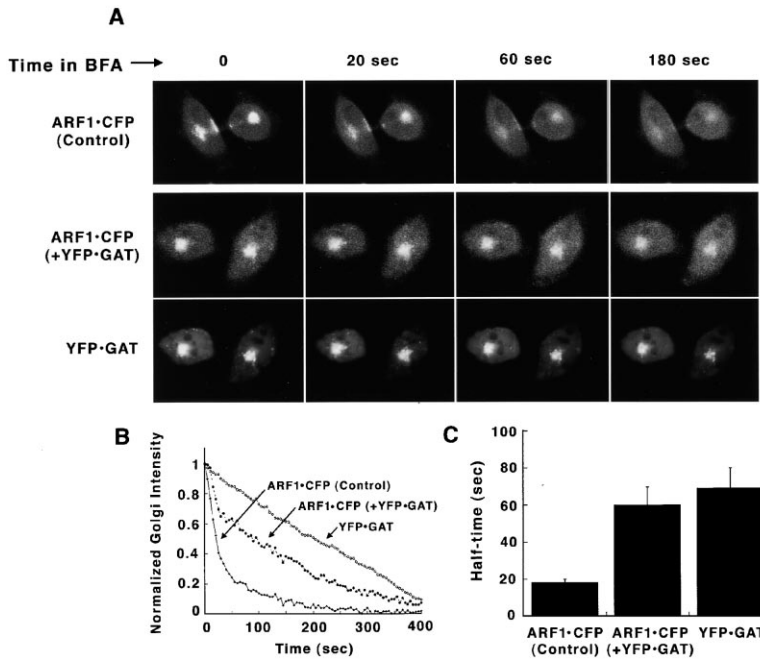


Figure 5. Stabilization of Golgi-Bound ARF1-GTP by Overexpression of the GAT Domain In Vivo

(A) Selected frames from time-lapse sequences in which brefeldin A (BFA) is added at time 0 are shown: (top) cells expressing ARF1-CFP alone; (middle) ARF1-CFP fluorescence in cells also expressing YFP-GAT; (bottom) YFP-GAT fluorescence in the same cells. (B) Quantification of cells shown in (A). Curves show fluorescence in a region of interest corresponding to the Golgi complex normalized such that 1 corresponds to initial fluorescence, while 0 is fluorescence at 600 s (when there was no longer any change in Golgi fluorescence). The curve labeled ARF1-CFP (Control) is from the right single-labeled cell in the corresponding strip in (A); the ARF1-CFP and YFP-GAT curves were obtained by quantifying the left of the two double-labeled cells.

(C) Average half-time of loss of ARF1-CFP or GAT-YFP from the Golgi apparatus after BFA addition (\pm standard error of the mean) in double-labeled cells ($n = 24$ cells) or in cells expressing ARF1-CFP only ($n = 14$ cells). Since many YFP-GAT expressing cells showed an initial rapid loss of 10%–30% ARF1-CFP from the Golgi (which may represent ARF1 not associated with the GAT domain), half-time shown is the time from 60% initial fluorescence (when this fast component was already lost) to 30% initial fluorescence.

Stabilization of Membrane-Bound ARF1-GTP by Expression of the GAT Domain In Vivo

Given that the GTP-bound form of ARF1 is associated with membranes while the GDP-bound form is cytosolic, the interference of GAT with GAP activity observed *in vitro* predicts that overexpression of the GAT domain should stabilize the association of ARF1 with membranes *in vivo*. To address this possibility, we measured the *in vivo* rate of dissociation from the Golgi complex of an ARF1-cyan fluorescent protein (CFP) construct in the absence or presence of a GGA3-GAT-yellow fluorescent protein (YFP) construct, both expressed by transient transfection into HeLa cells. Addition of the ARF-GEF inhibitor, brefeldin A, caused dissociation of ARF1-CFP from the Golgi complex with a half-time of ~ 20 s (Figures 5A–5C). When ARF1-CFP was coexpressed with GAT-YFP, the kinetics of ARF1-CFP dissociation became distinctly biphasic. A fraction of the ARF1-CFP molecules still dissociated at a rapid rate, while a second fraction dissociated with a half-time of ~ 60 s (Figures 5A–5C). This second fraction probably represents ARF1-CFP molecules complexed with GAT-YFP. It is noteworthy that the rate of dissociation of this second population of ARF1-CFP approached that of GAT-YFP (Figures 5A–5C), suggesting that release of GAT-YFP became the rate-limiting step for ARF-CFP dissociation from membranes. Thus, expression of GAT-YFP slows the dissociation of ARF-CFP from the Golgi complex, consistent with stabilization of membrane-bound ARF-GTP by interference with GAP activity.

Interaction of the GGAs with Clathrin In Vitro

The results presented thus far suggest that binding of the GGAs to membrane-associated ARF-GTP results in

transient stabilization of an ARF-GTP-GGA complex on the membrane. This stabilization may provide enough time for the GGAs to perform their function at the Golgi complex. But what might this function be? In a previous study (Dell'Angelica et al., 2000), we noted that the hinge domains of some of the GGAs contained putative clathrin binding motifs conforming to the L(L,I)(D,E,N)(L,F)(D,E) consensus (Goodman et al., 1997, Dell'Angelica et al., 1998, Ramjaun and McPherson, 1998). To analyze whether the GGAs actually bind clathrin, we performed GST pull-down assays using bovine brain cytosol as a source of clathrin. As positive controls in these assays, we used GST fusion proteins having the hinge-ear domains of $\beta 2$ adaptin (Shih et al., 1995) or a fragment spanning residues 337–523 of amphiphysin (Slepnev et al., 2000), both of which were predictably found to bind clathrin (Figure 6A). Also, as expected, GST, GST-VHS, or GST-VHS+GAT, did not bind clathrin in this assay (Figure 6A). In contrast, H+GAE domain constructs of GGA1, GGA2, and GGA3 all bound clathrin (Figure 6A). Separation of the hinge and GAE domains of the three GGAs revealed that the clathrin binding activity resided mainly within their hinge domains, although the GAE domain of GGA1 was also able to bind clathrin (Figure 6A). Mutation of the sequence LIDLE (residues 349–353) within the GGA2 hinge, which fits the consensus motif for clathrin binding (Dell'Angelica et al., 2000), to AADAA (GGA2H.A mutant) caused a marked decrease in clathrin binding. Likewise, mutation of the imperfect variant of this motif within the GGA1 hinge, LLDDE (residues 356–360), to AADAA (GGA1H.A mutant) also decreased clathrin binding. We also observed that a full-length GGA1 construct fused to GST bound clathrin (Figure 6A). Thus, these observations indicate that, like

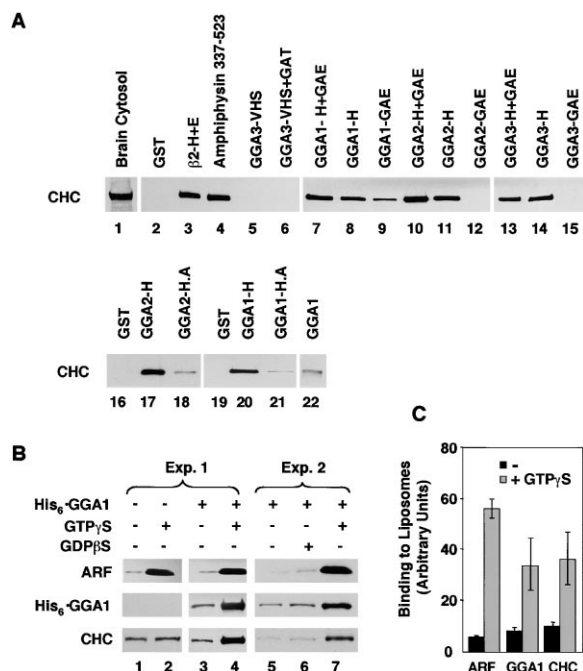


Figure 6. Interaction of GGAs with Clathrin In Vitro

(A) GST pull-down assays for clathrin binding. The hinge plus GAE domains (H+GAE), hinge alone (H), or GAE alone (GAE) of human GGA1, GGA2, and GGA3 fused to GST were tested for interaction with clathrin. GST, or GST fused to GGA3-VHS or GGA3-VHS+GAT were used as negative controls whereas GST fused to the hinge plus ear (H+E) domains of $\beta 2$ -adaptin or a fragment of amphiphysin (residues 337–523) were used as positive controls. A sequence fitting the consensus motif for clathrin binding (LIDLE) within the hinge domain of GGA2 and a related sequence (LIDDE) in the hinge domain of GGA1 were substituted by the sequence AADAA to generate the GGA2-H.A and GGA1-H.A constructs, respectively. Proteins were incubated with bovine brain cytosol and recovered with glutathione beads (see Experimental Procedures). Aliquots of the precipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Clathrin binding was detected with an antibody to the clathrin heavy chain (CHC). Equivalent amounts of GST fusion proteins, as determined by Coomassie blue staining of polyacrylamide gels, were loaded on all lanes except for lanes 13–15, containing GGA3 constructs. GGA3-fusion proteins were obtained in smaller amounts and their quantities on the gels determined by immunoblotting with anti-GST antibodies. The amounts loaded on the three lanes corresponding to GGA3 constructs, however, were equivalent.

(B) Recruitment to liposomes of ARF, His₆-GGA1, and clathrin in the absence (–) or presence of GTP γ S or GDP β S was analyzed as described in Experimental Procedures. The results of two experiments are shown.

(C) Quantification of ARF, His₆-GGA1 (GGA1), and clathrin binding to liposomes. Results are the mean \pm standard deviation of six determinations.

the $\beta 1$ -, $\beta 2$ -, and $\beta 3$ -adaptins (Shih et al., 1995; Dell'Angelica et al., 1998), the GGAs are able to bind clathrin in vitro via their hinge-GAE domains.

The ability of the GGAs to bind membrane-associated ARF1-GTP via the GAT domain and clathrin via the hinge-GAE domains suggests that they could promote recruitment of clathrin to membranes. To investigate this possibility, we incubated synthetic liposomes with bovine brain cytosol in the absence or presence of added recombinant His₆-GGA1, and in the absence or presence

of GTP γ S (Figure 6B). We observed that ARF was recruited to liposomes in a GTP γ S-dependent manner irrespective of the addition of His₆-GGA1 (Figures 6B and 6C). A 4.2 ± 1.3 -fold ($n = 6$) increase in the recruitment of His₆-GGA1 upon addition of GTP γ S was observed only in the His₆-GGA1-supplemented samples (Figure 6B, lanes 3 and 4, and Figure 6C). A small amount of clathrin was found to bind to liposomes in the absence of GTP γ S and His₆-GGA1 (Figure 6B, lane 1). This suggests that some clathrin can bind to liposomes in a GTP-independent fashion, as previously reported (Takei et al., 1998). Addition of GTP γ S resulted in a slight increase in clathrin binding (40% in the experiment shown in Figure 6B, lane 2), suggesting that adaptors present in the bovine brain cytosol are inefficient at enhancing clathrin binding under the conditions of these assays. However, addition of His₆-GGA1 to the cytosolic extract caused a 3.6 ± 1.1 -fold ($n = 6$) increase in the amount of clathrin recruited to liposomes in the presence of GTP γ S (Figure 6B, lanes 3 and 4, and Figure 6C) but not GDP β S (Figure 6B, compare lanes 5 and 6).

Functional Interactions of the GGAs with Clathrin

The experiments described above predict that GGAs could promote recruitment of clathrin to membranes in vivo. Indeed, transient transfection of HeLa cells with a myc-tagged GGA1 construct at moderate expression levels resulted in increased staining for clathrin in the area of the TGN (Figures 7A–7C, arrows). Quantification of this effect by image analysis revealed a 3.0 ± 0.6 -fold increase in cells expressing myc-tagged GGA1 relative to untransfected cells ($n = 19$ cells). Immunoelectron microscopy of transfected HeLa cells expressing high levels of GGA3 revealed a proliferation of coated vesicles and/or buds in the area of the TGN, many of which contained both GGA3 and clathrin (Figure 7P). Quantification of sections from different cells showed that 100 of 188 (53%) coated structures containing GGA3 (10 nm gold) also contained clathrin (15 nm gold) (Figure 7P, arrows).

Since both the hinge and GAE domains of GGA1 bind clathrin (Figure 6A), we reasoned that a truncated GGA1 construct lacking those domains could behave as a dominant-negative mutant in vivo. We found that this was the case, as expression of moderate levels of a myc-tagged GGA1 VHS+GAT construct in HeLa cells resulted in a marked decrease in clathrin staining at the TGN (Figures 7D–7F, arrows). Association of clathrin with the plasma membrane (Figures 7D–7F, arrows, peripheral punctate staining) and COPI with the Golgi complex (Figures 7G–7I, arrows), on the other hand, were not visibly affected by the expression of similar levels of GGA1 VHS+GAT. We also examined the effect of the GGA1 VHS+GAT construct on the distribution of the cation-independent mannose 6-phosphate receptor, which is sorted from the TGN to endosomes in a clathrin-dependent fashion (Liu et al., 1998). We observed that expression of GGA1 VHS+GAT caused a striking accumulation of the cation-independent mannose 6-phosphate receptor at the TGN and its concomitant depletion from the peripheral cytoplasm (Figures 7J–7L, arrows). In contrast, the distribution of the transferrin receptor was not affected (Figures 7M–7O, arrows). These obser-

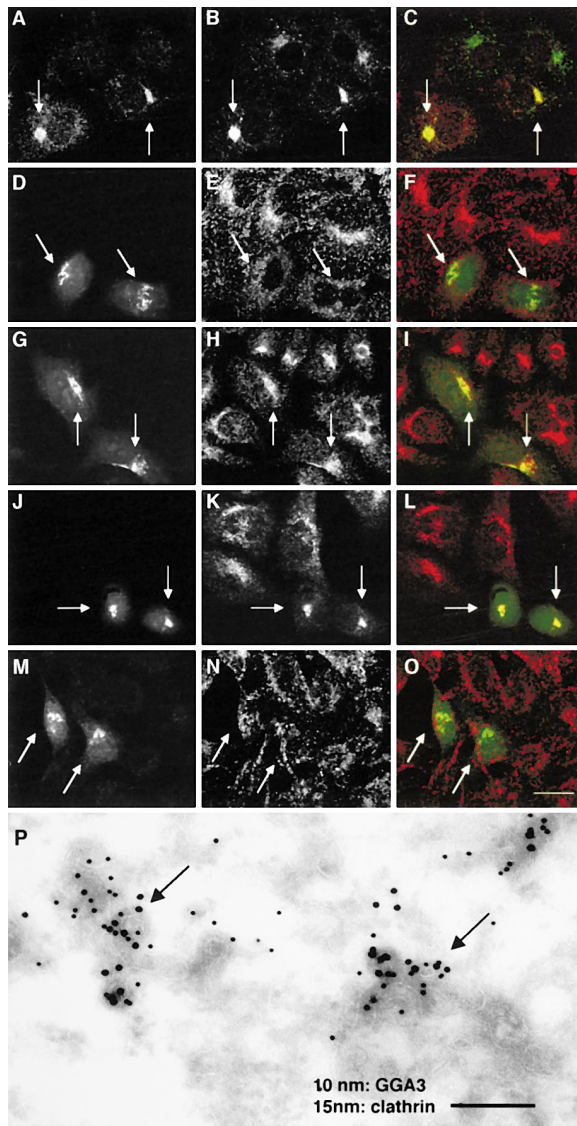


Figure 7. Effects of Full-Length and Truncated GGAs on Clathrin Recruitment to the TGN In Vivo

(A–C) HeLa cells were transfected with a plasmid encoding myc-tagged GGA1 and stained with rabbit polyclonal antibody to the myc epitope (A) and mouse monoclonal antibody to clathrin followed by Cy3-conjugated anti-rabbit IgG and fluorescein-conjugated anti-mouse IgG (B).

(D–O) HeLa cells were transfected with a plasmid encoding myc-tagged GGA1 VHS+GAT and stained with rabbit polyclonal antibody to the myc epitope (D, G, J, and M) and mouse monoclonal antibodies to clathrin (E), β-COP (H), cation-independent mannose 6-phosphate receptor (K), or transferrin receptor (N), followed by fluorescein-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG. The third image in each row is a merge of the corresponding first and second images. Arrows point to transfected cells. Bar, 20 μm. (P) Immunoelectron microscopy of HeLa cells transfected with a GGA3 construct and immunolabeled for GGA3 (10 nm gold) and clathrin (15 nm gold). Arrows point to coated structures containing both GGA3 and clathrin. Bar, 250 nm.

variations suggested that GGA1 VHS+GAT elicited a specific blockade in the clathrin-dependent transport of the cation-independent mannose 6-phosphate receptor from the TGN to endosomes.

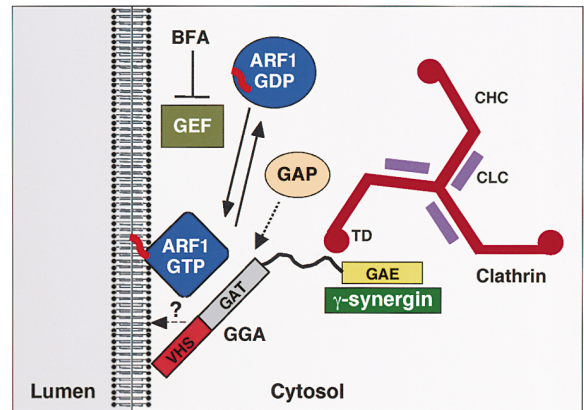


Figure 8. Model for the Assembly of GGA-Containing Coats

See “Discussion” for explanation of the model. CHC, clathrin heavy chain; CLC, clathrin light chain; TD, terminal domain.

Discussion

The experiments presented here show that GAT binding to ARF1 interferes with GAP activity and thus causes transient stabilization of ARF1-GTP on Golgi membranes. This phenomenon could contribute to the assembly of GGA-containing coats as depicted in the model shown in Figure 8. ARF-GDP is converted to ARF-GTP by the action of a Golgi-associated GEF. ARF-GTP then attaches to Golgi membranes via a myristoylated α helix at its amino terminus (Randazzo et al., 1995; Antonny et al., 1997). In the absence of effector binding, ARF-GTP is quickly acted on by a GAP, which converts it to ARF-GDP and causes its release from the membrane (Donaldson and Jackson, 2000). Binding of a GGA via its GAT domain hinders interaction of the GAP with ARF-GTP, resulting in transient stabilization of membrane-bound ARF-GTP. Effector-mediated increase in the affinity of ARF for GTP (Zhu et al., 2000) could also contribute to this stabilization. Clathrin would then be recruited to the membrane-bound ARF-GTP-GGA complex by virtue of an interaction between the clathrin terminal domain and the hinge and GAE domains of the GGA. The VHS domain could bind other proteins and/or membrane phospholipids (Misra et al., 2000). The GAE domain has been shown to interact with γ-synergins and other cytosolic proteins that might regulate coat assembly or vesicle budding (Hirst et al., 2000; Takatsu et al., 2000). According to this model, dissociation of GGAs from ARF-GTP would be a prerequisite for the GAP to be able to act on ARFs and induce its release from membranes.

This model differs from that postulated for the ARF-mediated recruitment of COPI onto membranes. Instead of interfering with GAP action, COPI enhances GAP-induced GTPase activity (Goldberg, 1999; but see also Szafer et al., 2000). This is probably due to the fact that COPI and ARF GAP1 bind to distinct, nonoverlapping sites on the ARF1 molecule (Goldberg, 1999), allowing for the formation of a tripartite ARF1-COPI-GAP complex with high hydrolytic activity for GTP. Binding of the GGAs involves both the switch 1 and switch 2 regions of ARF1 (this study; see also Kuai et al., 2000), while ARF GAP1 binds mainly to switch 2 and helix α3 of ARF1 (Goldberg,

1999). Thus, the competitive interactions between GGAs and GAPs could be due to steric hindrance at the level of switch 2. Competitive interactions between effectors and GAPs are in fact a characteristic of other members of the Ras superfamily of GTP binding proteins. For example, Rabphilin 3A (a Rab3A effector) and Rab GAP compete for binding to Rab3A (Kishida et al., 1993; Clabecq et al., 2000). Likewise, the sites for binding of Raf1 kinase (an effector of Ras) and Ras GAP to Ras are overlapping (Nassar et al., 1995; Scheffzek et al., 1997). Thus, COPI and the GGAs appear to affect the action of ARF GAPs differently, suggesting that they represent distinct modes of ARF regulation by coat proteins.

The GGAs have certain characteristics reminiscent of AP complexes. Some of these complexes bind to membranes in an ARF-dependent manner (Robinson and Kreis, 1992; Stamnes and Rothman, 1993; Ooi et al., 1998; Zhu et al., 1999a) and mediate recruitment of clathrin to membranes (Virshup and Bennett, 1988; Traub et al., 1995; Zhu et al., 1999b; Drake et al., 2000) by virtue of clathrin binding sites within the hinge and ear domains of their β subunits (Shih et al., 1995; Traub et al., 1995; Dell'Angelica et al., 1998; Owen et al., 2000). Unlike AP complexes, however, the GGAs are apparently monomeric (Dell'Angelica et al., 2000; Hirst et al., 2000). The GGAs are also not enriched in purified preparations of clathrin-coated vesicles (Hirst et al., 2000). In this regard, the GGAs might behave more like ARFs (Zhu et al., 1998) and other accessory factors (e.g., epsin 1, Chen et al., 1998) in that they may participate in clathrin coat assembly but are not major constituents of the resulting vesicles. The GGAs could thus function to prime membranes for clathrin recruitment, after which AP complexes such as AP-1 could intercalate into the forming coats. This would be followed by dissociation of the GGAs from ARFs, GAP-induced hydrolysis of GTP on ARFs, and consequent dissociation of ARFs from membranes.

Alternatively, the absence of the GGAs from purified preparations of clathrin-coated vesicles could be due to dissociation during homogenization and subcellular fractionation. In this case, the GGAs could function as conventional clathrin adaptors. Indeed, disruption of all three AP complexes in *S. cerevisiae* (i.e., AP-1, AP-2, and AP-3, of which only AP-1 appears to interact with clathrin), plus two AP180 homologs, does not prevent formation of clathrin-coated vesicles (Huang et al., 1999; Yeung et al., 1999). Two GGAs have been described in *S. cerevisiae*: Gga1p and Gga2p. Like their human counterparts, the *S. cerevisiae* GGAs have potential clathrin binding motifs in their hinge regions (Dell'Angelica et al., 2000). The *S. cerevisiae* GGAs have been implicated in sorting of CPY to the vacuole (Dell'Angelica et al., 2000; Hirst et al., 2000), a pathway that also involves clathrin (Seeger and Payne, 1992). It is thus tempting to speculate that the GGAs may contribute to clathrin-coated vesicle formation at the TGN, even in the absence of other AP complexes.

The role of the human GGAs in clathrin recruitment to the TGN is underscored by the enhanced association of clathrin with the TGN upon expression of moderate levels of GGA1. Conversely, moderate expression levels of a dominant-negative GGA1 construct lacking the clathrin binding domains causes specific dissociation

of clathrin from the TGN. Taken together, these observations are consistent with the notion that the GGAs function as ARF-dependent adaptors for clathrin recruitment to the TGN.

Experimental Procedures

Recombinant DNA Procedures

GFP-fusion constructs comprising different regions of the GGA3-GAT domain were prepared by PCR-amplification of codons 147–313, 147–260, 260–313, 170–233, 170–209, and 209–233, and subsequent cloning into the EcoRI-Sall sites of pEGFP-C2-MCS (Clontech, Palo Alto, CA). Segments comprising the GAT region of GGA1 (148–314), GGA2 (164–330), and TOM1 (215–299), and full-length GGA3 were also fused to GFP by PCR-amplification and cloning into pEGFP-C2-MCS (amino acid numbers indicated in parentheses). Point mutations in the GGA3-GAT region (D189A, N194A, and S199-T217P) and in full-length GGA3 (N199A) were introduced by PCR-based overlap extension. Constructs were cloned into the EcoRI and Sall sites of pEGFP-C2-MCS. The latter four constructs were also cloned into pGAD424 for two-hybrid assays and in pET-28a(+) (Novagen, Madison, WI) for production of His₆-tagged proteins. In addition, the VHS+GAT domains of GGA3 and full-length GGA1 were cloned into the pET-28a(+) vector via EcoRI-Sall restriction sites. The GAT region of GGA3 was also cloned into the Sall and BamHI sites of the pEYFP-C1 vector (Clontech). Two-hybrid ARF1 Q71L second site mutants were engineered using the overlap extension technique and cloned into the EcoRI-Sall sites of pGBT9 (Clontech). ARF1-CFP was constructed by cloning an EcoRI-BamHI fragment encoding full-length ARF1 into the multiple cloning site of pECFP-N1 (Clontech). Constructs encoding GST-GGA1-H+GAE (315–639), GST-GGA1-H (315–514), GST-GGA1-GAE (515–639), GST-GGA3-H+GAE (314–723), GST-GGA3-H (314–598), and GST-GGA3-GAE (599–723) were obtained by PCR-amplification and cloning into the EcoRI-Sall sites of pGEX-5X-1 (Pharmacia Biotech, Piscataway, NJ). The constructs GST-GGA2-H+GAE (331–613), GST-GGA2-H (331–488), and GST-GGA2-GAE (489–613) were also generated by PCR-amplification and cloning into the BamHI-Sall sites of pGEX-5X-1. GST-VHS+GAT and GST-H+E were described previously (Dell'Angelica et al., 1998, 2000). The GST-amphiphysin 337–523 was a gift of E. Dell'Angelica (UCLA).

Fluorescent Imaging of Live Cells

HeLa cells expressing constructs tagged with GFP spectral variants were grown on LabTek chambers (Nalge Nunc, Naperville, IL) containing RPMI 1640 medium supplemented with 25 mM HEPES and 20% (v/v) fetal bovine serum. Experiments were performed using an inverted confocal laser scanning microscope (Carl Zeiss LSM 510) equipped with a stage heated to 37°C, Argon and Krypton lasers, and a 63 \times 1.4 NA Planapochromat oil immersion objective. Time-lapse sequences were taken at 5 s intervals for 5–10 min. The ECFP and EYFP spectral variants of EGFP were distinguished in double-labeling experiments using the configuration described by Ellenberg et al. (1999).

ARF GAP Assays

ARF1 was loaded with [α -³²P]GTP in a mixture containing 4 μ M myristoylated ARF1, 25 mM HEPES (pH 7.4), 100 mM NaCl, 0.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, 10 μ M [α -³²P]GTP (specific activity: 10,000–50,000 cpm/pmol), and 500 μ M liposomes (60% phosphatidylcholine, 20% phosphatidylethanolamine, and 20% phosphatidylserine; Randazzo et al., 1992). Loading proceeded for 45 min at 30°C and was terminated by the addition of 2 mM MgCl₂. Loading efficiency with respect to [α -³²P]GTP was typically 60%–75%. GAP assays containing 40 nM [α -³²P]GTP-loaded ARF1 (approximately 1:10 dilution of the loading reaction) were performed with two different GAPs. 1.4 nM PZA fragment of ASAP1 (Brown et al., 1998) was assayed in a buffer containing 90 μ M PtdIns(4,5)P₂ (PIP₂) and 380 μ M phosphatidic acid in 0.1% (v/v) Triton X-100, 20 mM HEPES (pH 7.4), 100 mM NaCl, 1 mM GTP, 2 mM MgCl₂, and 1 mM dithiothreitol. In assays using ARF GAP1, the protein was purified from rat liver as previously described (Makler et al., 1995) and used at a concentration of 2.6 μ g/ml in a buffer that lacked PIP₂

and phosphatidic acid but had 0.1% (v/v) Triton X-100. Reactions were carried out for 2–4 min, and terminated by dilution into 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM MgCl₂, and 1 mM dithiothreitol at 4°C. The protein-bound guanine nucleotide was separated from free nucleotide by binding the protein to nitrocellulose filters (BA85, pore size 0.45 μm, size 25 mm, Schleicher and Schuell, Keene, NH) and the nucleotides released from the protein by extraction into 0.75–1 M formic acid. Finally, the samples were resolved into polyethylenimine (PEI) cellulose thin-layer chromatography (TLC) plates (Fisher Scientific) and the chromatogram was developed in 1 M formic acid and 1 M lithium chloride. GST- and His₆-tagged GGA constructs used in these and other assays were produced according to the manufacturer's instructions (Pharmacia Biotech and Clontech, respectively).

GST Pull-Down Assays

GST fusion proteins were tested for their ability to bind clathrin from bovine brain cytosol. Bovine brains were homogenized in 3 vol ice-cold 25 mM HEPES-KOH (pH 7.7), 250 mM sucrose, and 2 mM EDTA supplemented with 1 mM 1-(4-(2-aminoethyl)-benzenesulfonyl) fluoride hydrochloride, 10 μg/ml aprotinin, 3 μg/ml leupeptin, and 1.5 μg/ml pepstatin A. Cytosol was prepared from the homogenate by sequential centrifugation at 3,000 g for 10 min, 12,000 g for 20 min, and 100,000 g for 60 min at 4°C and stored in small aliquots at –80°C. GST fusion proteins (0.2 mg per sample) bound to glutathione-Sepharose beads were incubated with 1 ml bovine brain cytosol (8 mg/ml of protein) for 3 hr at 4°C. The beads were spun and washed three times with cold PBS. Beads were resuspended in SDS sample buffer and proteins resolved by electrophoresis on 4%–20% gradient gels, transferred onto nitrocellulose, and analyzed by immunoblotting using an anti-clathrin heavy chain antibody (CHC, Transduction Laboratories, Lexington, KY) or stained with Coomassie Blue for GST protein detection.

Coat-Recruitment Assays

Phosphatidylcholine (60%), phosphatidylserine (30%), and PIP2 (10%) (Sigma Chemical Co., St. Louis, MO) were dissolved in chloroform in glass tubes. The chloroform was removed with a stream of nitrogen, and the dry film of the lipids was sonicated into 20 mM HEPES (pH 7.4), 20 mM KCl, and 0.2 M sucrose. Shortly before the coat-recruitment assay, the lipid vesicles were pelleted by a 15 min centrifugation at 75,000 rpm in a TLA100.3 rotor (Beckman) and resuspended into assay buffer (20 mM HEPES [pH 7.4], 100 mM NaCl, 0.5 mM MgCl₂, 1 mM EDTA, and 1 mM DTT) supplemented with an ATP-regenerating system consisting of 1 mM ATP, 5 mM creatine phosphate, and 10 U/ml creatine kinase. In a typical 400 μl reaction, 150 μl binding buffer was incubated with 100 μl liposomes (1 mM) and 200 μl bovine brain cytosol (8 mg protein/ml) in the presence or absence of 0.1 mM GTPγS and 0.5 mg/ml recombinant His₆GGA1 full-length protein. Incubations were carried out for 30 min at 30°C, and the reaction mixture was centrifuged for 15 min at 75,000 rpm. The vesicles were resuspended in SDS sample buffer and resolved by SDS-PAGE and immunoblotting.

Other Procedures and Reagents

Cell culture and transfection, immunofluorescence and immunoelectron microscopy, and yeast two-hybrid assays were performed as previously described (Dell'Angelica et al., 2000). The following antibodies were used in these studies: mouse monoclonal antibody to γ1-adaptin (100/3, Sigma Chemical Co., St. Louis, MO); mouse monoclonal antibody to clathrin (CHC); mouse monoclonal antibody to the human transferrin receptor (B3/25, Boehringer, Indianapolis, IN); mouse monoclonal antibody to the cation-independent mannose 6-phosphate receptor (kind gift from S. Pfeffer, Stanford University, CA); and rabbit polyclonal antibody to β-COP (kind gift of J. Lippincott-Schwartz, NIH, Bethesda, MD).

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References

- Antonny, B., Beraud-Dufour, S., Chardin, P., and Chabre, M. (1997). N-terminal hydrophobic residues of the G-protein ADP-ribosylation factor-1 insert into membrane phospholipids upon GDP to GTP exchange. *Biochemistry* 36, 4675–4684.
- Black, M.W., and Pelham, H.R. (2000). A selective transport route from Golgi to late endosomes that requires the yeast GGA proteins. *J. Cell Biol.* 151, 587–600.
- Boman, A.L., Zhang, C.J., Zhu, X., and Kahn, R.A. (2000). A family of ADP-ribosylation factor effectors that can alter membrane transport through the trans-Golgi. *Mol. Biol. Cell* 11, 1241–1255.
- Brown, M.T., Andrade, J., Radhakrishna, H., Donaldson, J.G., Cooper, J.A., and Randazzo, P.A. (1998). ASAP1, a phospholipid-dependent arf GTPase-activating protein that associates with and is phosphorylated by Src. *Mol. Cell. Biol.* 18, 7038–7051.
- Chen, H., Fre, S., Slepnev, V.I., Capua, M.R., Takei, K., Butler, M.H., Di Fiore, P.P., and De Camilli, P. (1998). Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* 394, 793–797.
- Clabecq, A., Henry, J.P., and Darchen, F. (2000). Biochemical characterization of Rab3-GTPase Activating Protein reveals a mechanism similar to that of Ras-GAP. *J. Biol. Chem.* 275, 31786–31791.
- Cukierman, E., Huber, I., Rotman, M., and Cassel, D. (1995). The ARF1 GTPase-activating protein: zinc finger motif and Golgi complex localization. *Science* 270, 1999–2002.
- Dell'Angelica, E.C., Klumperman, J., Stoorvogel, W., and Bonifacino, J.S. (1998). Association of the AP-3 adaptor complex with clathrin. *Science* 280, 431–434.
- Dell'Angelica, E.C., Puertollano, R., Mullins, C., Aguilar, R.C., Vargas, J.D., Hartnell, L.M., and Bonifacino, J.S. (2000). GGAs: a family of ADP ribosylation factor-binding proteins related to adaptors and associated with the Golgi complex. *J. Cell Biol.* 149, 81–94.
- Donaldson, J.G., and Jackson, C.L. (2000). Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.* 12, 475–482.
- Drake, M.T., Zhu, Y., and Kornfeld, S. (2000). The assembly of AP-3 adaptor complex-containing clathrin-coated vesicles on synthetic liposomes. *Mol. Biol. Cell* 11, 3723–3736.
- Ellenberg, J., Lippincott-Schwartz, J., and Presley, J.F. (1999). Dual-colour imaging with GFP variants. *Trends Cell Biol.* 9, 52–56.
- Goldberg, J. (1999). Structural and functional analysis of the ARF1-ARFGAP complex reveals a role for coatamer in GTP hydrolysis. *Cell* 96, 893–902.
- Goodman, O.B., Jr., Krupnick, J.G., Gurevich, V.V., Benovic, J.L., and Keen, J.H. (1997). Arrestin/clathrin interaction. Localization of the arrestin binding locus to the clathrin terminal domain. *J. Biol. Chem.* 272, 15017–15022.
- Hirst, J., Lui, W.W., Bright, N.A., Totty, N., Seaman, M.N., and Robinson, M.S. (2000). A family of proteins with gamma-adaptin and VHS domains that facilitate trafficking between the trans-Golgi network and the vacuole/lysosome. *J. Cell Biol.* 149, 67–80.
- Huang, K.M., D'Hondt, K., Riezman, H., and Lemmon, S.K. (1999). Clathrin functions in the absence of heterotetrameric adaptors and AP180-related proteins in yeast. *EMBO J.* 18, 3897–3908.
- Kishida, S., Shirataki, H., Sasaki, T., Kato, M., Kaibuchi, K., and Takai, Y. (1993). Rab3A GTPase-activating protein-inhibiting activity of Rabphilin-3A, a putative Rab3A target protein. *J. Biol. Chem.* 268, 22259–22261.
- Kuai, J., Boman, A.L., Arnold, R.S., Zhu, X., and Kahn, R.A. (2000). Effects of activated ADP-ribosylation factors on Golgi morphology require neither activation of phospholipase D1 nor recruitment of coatamer. *J. Biol. Chem.* 275, 4022–4032.
- Liu, S.H., Marks, M.S., and Brodsky, F.M. (1998). A dominant-negative clathrin mutant differentially affects trafficking of molecules with

- distinct sorting motifs in the class II major histocompatibility complex (MHC) pathway. *J. Cell. Biol.* 140, 1023–1037.
- Makler, V., Cukierman, E., Rotman, M., Admon, A., and Cassel, D. (1995). ADP-ribosylation factor-directed GTPase-activating protein. Purification and partial characterization. *J. Biol. Chem.* 270, 5232–5237.
- Misra, S., Beach, B.M., and Hurley, J.H. (2000). Structure of the VHS domain of human tom1 (target of myb 1): insights into interactions with proteins and membranes. *Biochemistry* 39, 11282–11290.
- Nassar, N., Horn, G., Herrmann, C., Scherer, A., McCormick, F., and Wittinghofer, A. (1995). The 2.2 Å crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue. *Nature* 375, 554–560.
- Ooi, C.E., Dell'Angelica, E.C., and Bonifacino, J.S. (1998). ADP-ribosylation factor 1 (ARF1) regulates recruitment of the AP-3 adaptor complex to membranes. *J. Cell Biol.* 142, 391–402.
- Owen, D.J., Vallis, Y., Pearse, B.M., McMahon, H.T., and Evans, P.R. (2000). The structure and function of the beta2-adaptin appendage domain. *EMBO J.* 19, 4216–4227.
- Poussu, A., Lohi, O., and Lehto, V.P. (2000). Vear, a novel Golgi-associated protein with VHS and gamma-adaptin "ear" domains. *J. Biol. Chem.* 275, 7176–7183.
- Ramjaun, A.R., and McPherson, P.S. (1998). Multiple amphiphysin II splice variants display differential clathrin binding: identification of two distinct clathrin-binding sites. *J. Neurochem.* 70, 2369–2376.
- Randazzo, P.A., Weiss, O., and Kahn, R.A. (1992). Preparation of recombinant ADP-ribosylation factor. *Methods Enzymol.* 219, 362–369.
- Randazzo, P.A., Terui, T., Sturch, S., Fales, H.M., Ferrige, A.G., and Kahn, R.A. (1995). The myristoylated amino terminus of ADP-ribosylation factor 1 is a phospholipid- and GTP-sensitive switch. *J. Biol. Chem.* 270, 14809–14815.
- Robinson, M.S., and Kreis, T.E. (1992). Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell* 69, 129–138.
- Roth, M.G. (1999). Snapshots of ARF1: implications for mechanisms of activation and inactivation. *Cell* 97, 149–152.
- Scheffzek, K., Ahmadian, M.R., Kabsch, W., Wiesmuller, L., Lautwein, A., Schmitz, F., and Wittinghofer, A. (1997). The Ras-RasGAP complex: structural basis for GTPase activation and its loss in oncogenic Ras mutants. *Science* 277, 333–338.
- Seeger, M., and Payne, G.S. (1992). A role for clathrin in the sorting of vacuolar proteins in the Golgi complex of yeast. *EMBO J.* 11, 2811–2818.
- Shih, W., Gallusser, A., and Kirchhausen, T. (1995). A clathrin-binding site in the hinge of the beta 2 chain of mammalian AP-2 complexes. *J. Biol. Chem.* 270, 31083–31090.
- Slepnev, V.I., Ochoa, G.C., Butler, M.H., and De Camilli, P. (2000). Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites. *J. Biol. Chem.* 275, 17583–17589.
- Stamnes, M.A., and Rothman, J.E. (1993). The binding of AP-1 clathrin adaptor particles to Golgi membranes requires ADP-ribosylation factor, a small GTP-binding protein. *Cell* 73, 999–1005.
- Szafer, E., Pick, E., Rotman, M., Zuck, S., Huber, I., and Cassel, D. (2000). Role of coatomer and phospholipids in GTPase-activating protein-dependent hydrolysis of GTP by ADP-ribosylation factor-1. *J. Biol. Chem.* 275, 23615–23619.
- Takatsu, H., Yoshino, K., and Nakayama, K. (2000). Adaptor gamma ear homology domain conserved in gamma-adaptin and GGA proteins that interact with gamma-synergins. *Biochem. Biophys. Res. Commun.* 271, 719–725.
- Takei, K., Haucke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H., and De Camilli, P. (1998). Generation of coated intermediates of clathrin-mediated endocytosis on protein-free liposomes. *Cell* 94, 131–141.
- Traub, L.M., Kornfeld, S., and Ungewickell, E. (1995). Different domains of the AP-1 adaptor complex are required for Golgi membrane binding and clathrin recruitment. *J. Biol. Chem.* 270, 4933–4942.
- Virshup, D.M., and Bennett, V. (1988). Clathrin-coated vesicle assembly polypeptides: physical properties and reconstitution studies with brain membranes. *J. Cell Biol.* 106, 39–50.
- Yeung, B.G., Phan, H.L., and Payne, G.S. (1999). Adaptor complex-independent clathrin function in yeast. *Mol. Biol. Cell* 10, 3643–3659.
- Zhdankina, O., Strand, N.L., Redmond, J.M., and Boman, A.L. (2001). Yeast GGA proteins interact with GTP-bound Arf and facilitate transport through the Golgi. *Yeast* 18, 1–18.
- Zhu, Y., Traub, L.M., and Kornfeld, S. (1998). ADP-ribosylation factor 1 transiently activates high-affinity adaptor protein complex AP-1 binding sites on Golgi membranes. *Mol. Biol. Cell* 9, 1323–1337.
- Zhu, Y., Drake, M.T., and Kornfeld, S. (1999a). ADP-ribosylation factor 1 dependent clathrin-coat assembly on synthetic liposomes. *Proc. Natl. Acad. Sci. USA* 96, 5013–5018.
- Zhu, Y., Traub, L.M., and Kornfeld, S. (1999b). High-affinity binding of the AP-1 adaptor complex to trans-golgi network membranes devoid of mannose 6-phosphate receptors. *Mol. Biol. Cell* 10, 537–549.
- Zhu, X., Boman, A.L., Kuai, J., Cieplak, W., and Kahn, R.A. (2000). Effectors increase the affinity of ADP-ribosylation factor for GTP to increase binding. *J. Biol. Chem.* 275, 13465–13475.